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Developmental and environmental stability of candidate reference genes in the wild bee *ceratina calcarata*

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Quantitative real-time PCR (q-RT-PCR) is a widely used method for measuring gene expression, but its accuracy depends on the use of stable reference genes for data normalization. In this study, we evaluated the expression stability of seven candidate reference genes (*RPS18*, *RPS5*, *RPL32*, *RPL8*, *EF-1α*, *β-Actin*, and *GAPDH*) in the small carpenter bee *Ceratina calcarata* across developmental stages (larvae, pupae, adults) and different landscape environments (conventional farms, organic farms, and roadside sites). Using four analytical algorithms, GeNorm, NormFinder, BestKeeper, and the comparative ΔCt method, we identified *RPS18* and *RPL8* as the most stable reference genes under varying biological and environmental conditions. These findings were further supported by RefFinder, which integrates results from all algorithms. Our study provides the first validated reference genes for *C. calcarata*, enabling more accurate and reproducible gene expression analysis in this ecologically important wild bee species. This work will support future research in pollinator biology, environmental stress responses, and conservation genomics.

Keywords Small carpenter bee, Gene expression stability, Q-RT-PCR, Reference gene, RPS18, RPL8

The service provided by pollinators is essential for global ecosystems. About 87.5% of flowering plants worldwide require pollination service by animals[1]. About 75% of the agricultural crops benefit from pollinators, resulting in a 35% increase in yield and generating billions of dollars in value[2,3]. The honeybee (*Apis mellifera*) is the most important managed pollinator for crop and fruit productions[4,5]. Native wild bees are far more numerous than managed bees[6]. They provide complementary pollination service thought to surpass managed bees, which contribute to the ecological and agricultural stability[7,8]. The small carpenter bees refer to a large group of more than 300 species from genus *Ceratina* (Apidae: Xylocopinae)[9]. *Ceratina calcarata* Robertson is a species native to eastern North America, ranging Florida to the south, Ontario to the north, and Nova Scotia to the east[10]. The species, as well as the sympatric species *C. dupla* Say, can effectively pollinate many fruit, vegetable, and other crops[11,12] and are among the most abundant pollinator species in recent restored land[12,13]. Similar to honey bees, native wild bees also suffered from declining populations, potentially caused by loss of habitat, the use of agrochemicals, land-scape alternation, and parasitism[14,15].

Besides its economic and ecological importance, *C. calcarata* is considered as a subsocial species with features of prolonged maternal care and mother-adult offspring interaction. It also demonstrates traits of facultative sociality such as division of labor and cooperative brood care[16]. The eldest daughters are dwarf in body size and responsible for foraging, guarding and feeding their younger siblings[17–19]. Their roles resemble worker-like behavior in eusocial species and they do not have chance to overwinter or reproduce the next spring. Therefore, *C. calcarata* is an ideal model to study the evolution and mechanism of sociality in hymenopteran insects.

To date, many molecular and genomics approaches have been adopted to study the phylogeny[10,20,21], adaptation[22,23] and reproduction[24] of *Ceratina* species. The reference genome of *C. calcarata*[25] and *C. australensis*[26] has been sequenced and assembled. The transcriptome and metatranscriptome of *C. calcarata* have been sequenced, which revealed the gene and microbiome regulations associated with overwintering[27], maternal and sibling care[18,28,29], social behavior[30], and landscape adaptation[31]. Compared to high-throughput sequencing,

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real-time quantitative reverse transcription polymerase chain reaction (q-RT-PCR) provides a fast and accurate approach to quantify genes[32–34]. It involves the reverse transcription of RNA into complementary DNA (cDNA), followed by real-time PCR amplification. This method is cost-effective when a small number of target genes are analyzed from a large number of samples. The calculation of relative expression is relied on internal controls, which are housekeeping genes with constant expression levels across the treatments[35]. However, as a prerequisite procedure for studying gene expressions associated with development and adaptation to agricultural landscapes, there is a lack of highly conserved reference genes in *C. calcarata* for q-RT-PCRs. Thus, it is a challenge to study target gene expressions in this bee species.

In this study, we tested the expressional stability of seven commonly used reference genes, including four ribosomal binding proteins: ribosomal protein L8 (*RPL8*) and L32 (*RPL32*) binding to large subunits, S5 (*RPS5*) and S18 (*RPS18*) binding to small subunit, a cytoskeleton protein: β-actin (*ACT*), a translation elongation protein: elongation factor 1-alpha F2 (*EF-1α*), and a housekeeping enzyme: glyceraldehyde 3-phosphate dehydrogenase (*GADPH*). We used four methods to compare the stability of each gene: comparative ΔCt analysis[36], NormFinder[37], geNorm[38], and BestKeeper[39]. Further RefFinder was utilized for integrated analysis with incorporating GeNorm, BestKeeper, NormFinder, and ΔCt analysis[40]. Our results present the most stable genes across landscapes and developmental stages, which can be used in studies of gene expression analysis under similar scenarios.

Materials and methods

Sample collection and total RNA extraction

C. calcarata individuals were collected from multiple sites in Western Ohio during the spring 2024. The sites include three types of landscape: conventional farms with regular applications of pesticides and other agrochemicals, organic farms with natural based pesticides, and roadside landscape without agricultural activity. Given that *C. calcarata* nests in raspberry (*Rubus idaeus* L.)[17,41], raspberry stems with diameters over 7 mm from the previous growing season were cut to 1.20 m stems, attached to bamboo sticks with twist ties, and vertically inserted into the ground randomly for about 20 cm in early May to attract *C. calcarata* (Supplementary figure S1).

After four weeks, the nesting individuals were collected from stems and their life stages were visually identified as larva, pupa and adult stages. Immatures were categorized into small larvae, large larvae, pre-pupae, white-eyed pupae[31]. For RNA extraction, we used large larvae which may be either 4th or 5th instar. However, we did not determine the sex of the larvae, and sex differences may contribute to variation in gene expression. The samples were frozen in dry ice and stored temporarily at −80 °C. Individual total RNA was extracted using TRIzol and purified by ZYMO Direct-zol RNA Miniprep Kit (Zymo Research, Irvine, CA, USA). Genomic DNA (gDNA) was removed by DNase I (Zymo Research) using in-column digestion method. The concentrations of RNA were measured by Qubit RNA BR Assay (Thermo Fisher Scientific, Waltham, MA). The RNA samples were preserved at −80 °C for future use.

Primer design and q-RT-PCR experiments, and efficiency test

The predicted coding sequences of candidate genes were searched from *C. calcarata* genome assembly annotation[25] and confirmed by PCR. The primers were designed using Primer3Plus with default settings with q-RT-PCR module targeting amplicons of 90–130 bp[42]. The details of the primers are presented in Supplementary Table S1. Where possible, primers were designed to span exon-exon junctions to avoid amplification of gDNA. Primer specificity was confirmed by reverse transcription polymerase chain reaction (RT-PCR) followed by 1% agarose gel electrophoresis, which produced a single band of the expected size for each primer set. A single peak in the melting curve further verified the specificity of the amplification. Primer efficiency was determined using a five-point, 10-fold serial dilution of pooled cDNA as standard. The amplification efficiencies ranged from 91.05% to 108.40%.

The first strand cDNA for each sample was synthesized by iScript cDNA Synthesis Supermix (Bio-Rad, Hercules, CA) following the factory protocol. A mixture of oligo(dT) and random hexamers were used to prime the reaction. Samples were normalized to 1 µg total RNA per 20 µl reaction mix. Real time PCR were conducted using PowerUp SYBR Green Mix (Thermo Fisher Scientific) in QuantStudio 3 Real-Time PCR System (Thermo Fisher Scientific) by following procedure: 50 °C for 2 min, 95 °C for 2 min; 40 cycles of 95 °C for 15 s, 60 °C for 30 s; then a 60–95 °C melting curve to confirm the specificity of amplification. Three technical replications were incorporated for each sample. To obtain the PCR efficiency of each primer set, q-RT-PCR was also performed on 10X serial dilution of cDNA. The efficiency of each primer set was calculated by formula $Efficiency = 10^{-1/slope}$.

Data analysis

The cycle threshold (Ct) value of each reaction was obtained by Design & Analysis 2 (DA2) software (version 2.8.0, Thermo Fisher Scientific). Standard curves of Ct were made by liner regression, and the efficiency of each primer set was calculated by formula as mentioned above. We addressed the expression stabilities under developmental stages or agricultural landscapes using following algorithms: comparative ΔCt method[36], NormFinder[37], geNorm[38], and BestKeeper[39]. R Package ctrlGene (version 1.0.1)[43] were used to address geNorm and BestKeeper analysis. RefFinder was utilized for integrated analysis with incorporating GeNorm, BestKeeper, NormFinder, and ΔCt analysis[40].

Results

Primer specificity and efficiency test

The specificity of primers was confirmed by reverse transcription PCR. The results of 1% agarose gel electrophoresis followed presented the unique bands for each primer set within the expected size range. The single peak in melting curves also confirmed the result. Using cDNA serial dilution as standard, the primers present efficiency from 91.05% to 108.40%, which are within the acceptable range.

Analysis of candidate reference gene expression

The expression levels of different reference genes of *C. calcarata* under different developmental stages and landscapes is presented in the Fig. 1a, b. The results revealed a range of Ct values for analysed candidate reference genes varying from 14.83 to 32.01. The Ct values for candidate reference genes varied across developmental stages (larvae vs. adult) and collection sites (conventional vs. organic vs. roadside sites). Among all genes, *RPS18* and *RPL8* showed lower Ct variability. However, *GADPH* and *ACT* exhibited greater fluctuation.

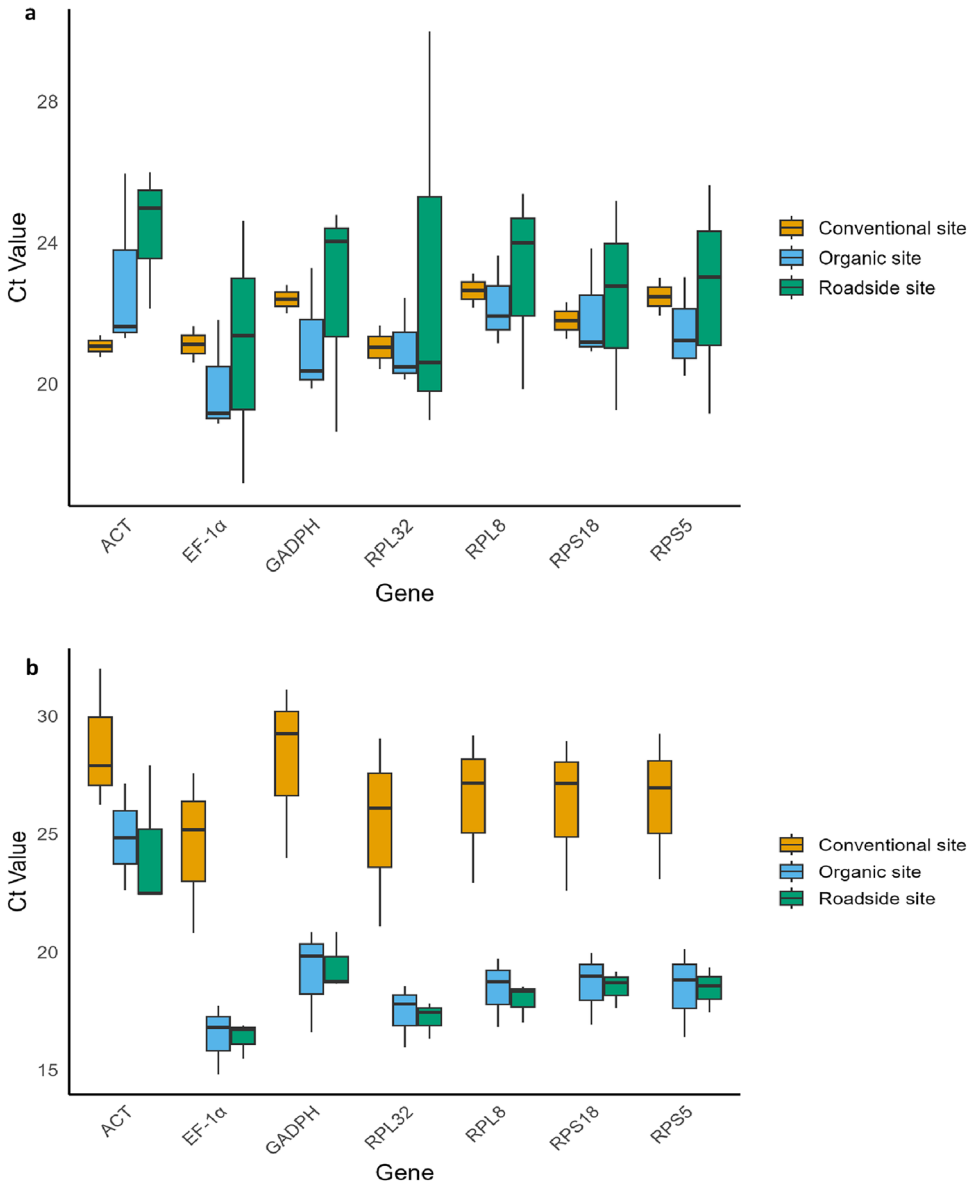

Fig. 1. Expression levels of different candidate reference genes in small carpenter bee across developmental stages and landscapes. The x-axis shows the tested candidate reference genes and the y-axis shows their relative expression levels (mean standard deviation of Ct values. (**a**) Adult stage samples (**b**) Larval stage samples. Bars represent mean vaues from biological replicates; error bars indicate standard deviation.

Stability of candidate reference genes

ΔCt method

The ΔCt method utilizes the standard deviations of Ct values to assess gene expression stability of genes. In both larval and adult stages, *RPL8* was the most stable gene among the seven investigated genes. While analyzing the data based on collection sites as organic, roadside and conventional sites, *RPS18*, *RPS5* and *RPS32* were the more stable genes, respectively (Fig. 2a-e).

GeNorm analysis

GeNorm analysis uses expression stability measurement (M) value based on the average pairwise variation to calculate the stability of expression levels. The M of different candidate reference genes using geNorm are presented in Fig. 3a-e. *RPL8* followed by *RPL32* were the most stable genes in the larval stage, whereas *RPS18* followed by *RPL8* were the most stable genes in adult stage. In addition, *RPS18*, *RPS5* and *RPL8* were the most stable genes based on the organic, roadside and conventional sites of collection, respectively.

BestKeeper analysis

BestKeeper uses both the coefficient of variations (CVs) and standard deviations (SDs) to determine the stability of each candidate reference gene. The SDs used by BestKeeper were from Ct values. The stability of a reference gene is considered better if it has a lower CV ± SD value[44]. According to BestKeeper analysis, *ACT* and *RPS18* were the top two most sable genes in the larval stage and *RPS18* and *RPL8* were the top most stable genes in the adult stage. The most stable candidate reference genes identified were *RPL32* and *RPLS18* on the organic area, *RPS18* and *RPS5* on the roadside area, and *ACT* and *RPS18* on the conventional area (Table 1).

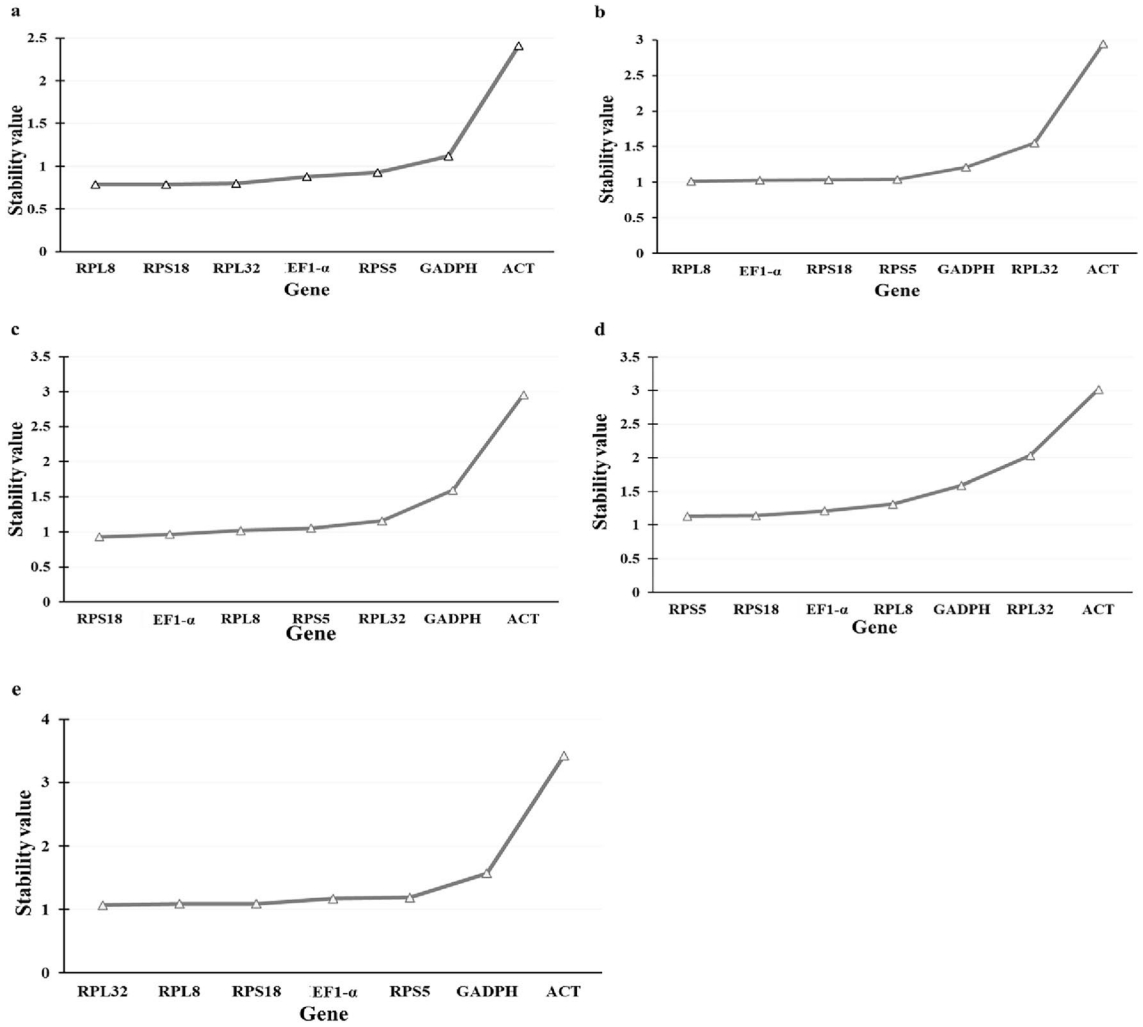

Fig. 2. Expression stability of candidate reference genes in small carpenter bee across different developmental stages and landscapes using ΔCt method. The x-axis shows the candidate reference genes, and y-axis shows mean ΔCt values, where lower values indicate higher expression stability. (**a**) Larval stage (**b**) Adult stage (**c**) Organic sites (**d**) Roadside sites (**e**) Conventional sites.

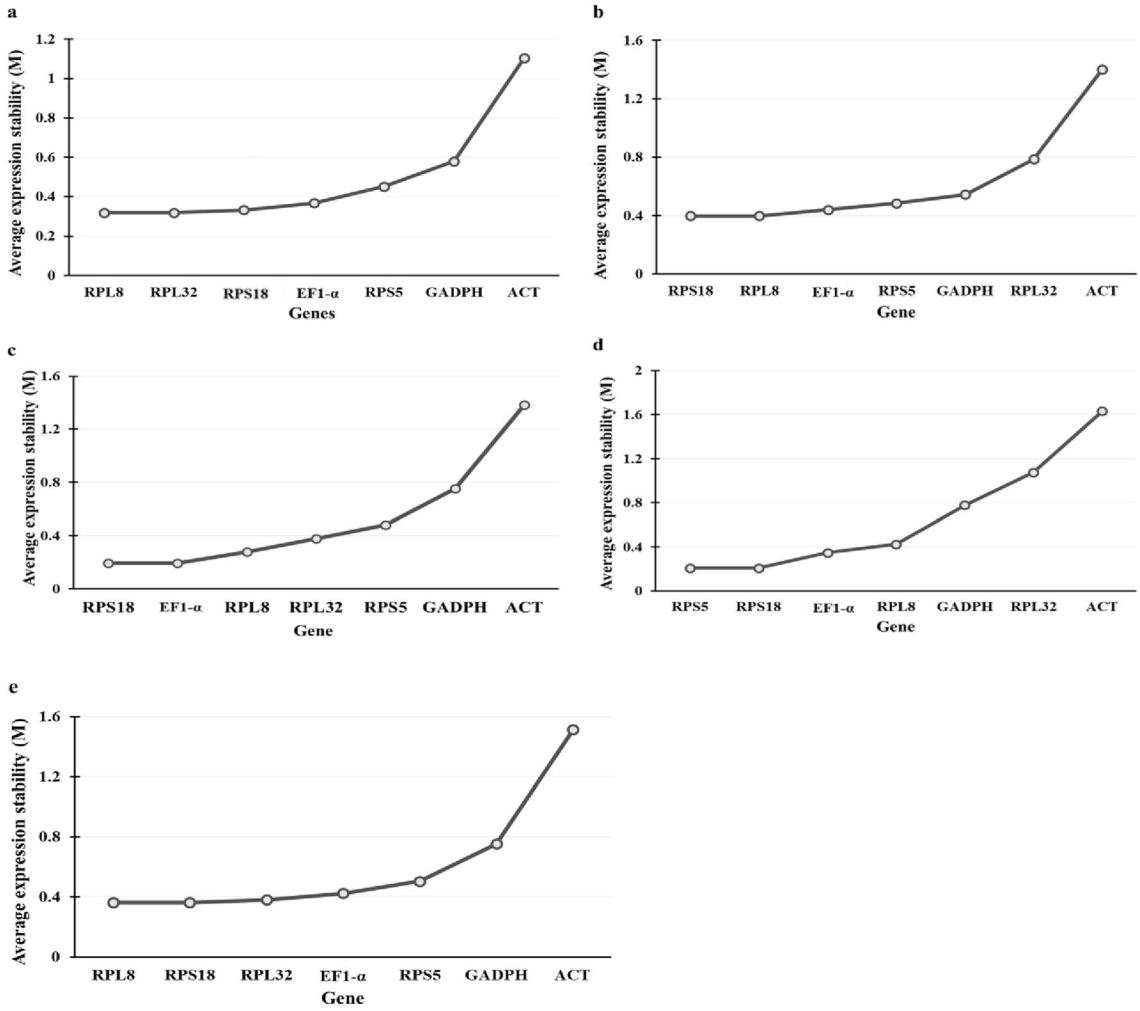

Fig. 3. Expression stability measurement (M value) of candidate reference genes in small carpenter bee across developmental stages and landscapes using geNorm analysis. The x-axis shows the candidate reference genes, and the y-axis shows their average expression stability value (M value), where lower M indicates higher stability. (**a**) Larval stage (**b**) Adult stage (**c**) Organic sites (**d**) Roadside sites (**e**) Conventional sites.

Larva		Adult		Organic site		Roadside site		Conventional site	
Gene	CV ± SD	Gene	CV ± SD	Gene	CV ± SD	Gene	CV ± SD	Gene	CV ± SD
ACT	8.53 ± 2.12	*RPS18*	8.42 ± 1.66	*RPL32*	5.67 ± 1.06	*RPS18*	7.69 ± 1.48	*ACT*	11.20 ± 2.59
RPS18	10.78 ± 2.02	*RPL8*	8.81 ± 1.77	*RPS18*	6.35 ± 1.24	*RPS5*	8.27 ± 1.59	*RPS18*	13.02 ± 2.48
RP8	11.48 ± 2.14	*RPL32*	9.41 ± 1.80	*RPL8*	6.54 ± 1.28	*RPL8*	8.76 ± 1.70	*RPL8*	14.09 ± 2.71
EF-1α	11.92 ± 2.01	*EF-1α*	10.13 ± 1.83	*RPS5*	6.72 ± 1.28	*GADPH*	9.14 ± 1.78	*RPL32*	15.13 ± 2.72
RPL32	12.23 ± 2.15	*RPS5*	10.13 ± 1.96	*EF-1α*	6.92 ± 1.21	*EF-1α*	9.71 ± 1.69	*EF-1α*	15.35 ± 2.68
RPS5	12.69 ± 2.34	*ACT*	10.2 ± 2.27	*GADPH*	8.26 ± 1.56	*ACT*	10.14 ± 2.36	*RPS5*	16.52 ± 2.99
GADPH	12.73 ± 2.53	*GADPH*	11.31 ± 2.14	*ACT*	9.16 ± 2.25	*RPL32*	10.82 ± 2.02	*GADPH*	16.52 ± 3.23

Table 1. Expression stability of candidate reference genes in small carpenter bee under different developmental stages and landscapes using bestkeeper analysis. CV: Coefficient of variation, SD: Standard deviation.

NormFinder analysis

NormFinder identifies the most suitable reference gene by an expression stability value, where lower values indicate more stable expression. The expression stability of candidate reference genes of *C. calcarata* under different developmental stages and landscapes using NormFinder is presented in Fig. 4. *RPS18* and *RPL32* were identified as the most stable candidate reference genes based on the NormFinder analysis.

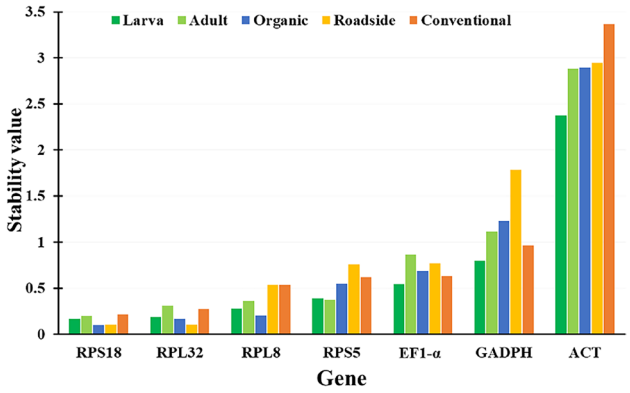

Fig. 4. Expression stability of reference genes in small carpenter bee across different developmental stages and landscapes using NormFinder analysis. The x-axis shows the candidate reference genes, and the y-axis shows their stability value calculated by NormFinder, where lower values indicate more stable expression.

Larva		Adult		Organic site		Roadside site		Conventional site	
Gene	Stability value	Gene	Stability value	Gene	Stability value	Gene	Stability value	Gene	Stability value
RPL8	1.68	*RPS18*	1.31	*RPS18*	1.31	*RPS5*	1.19	*RPS18*	1.32
RPS18	1.86	*RPL8*	1.68	*EF-1α*	1.86	*RPS18*	1.41	*RPL8*	2.21
RPL32	2.59	*EF-1α*	2.63	*RPL32*	3.16	*EF-1α*	3.00	*RPL32*	2.34
EF-1α	2.83	*RPS5*	3.93	*RPL8*	3.46	*RPL8*	4.00	*EF-1α*	3.94
RPS5	5.23	*RPL32*	5.04	*RPS5*	3.76	*GADPH*	5.00	*RPS5*	4.95
ACT	5.66	*GADPH*	5.23	*GADPH*	6.00	*RPL32*	6.00	*ACT*	5.12
GADPH	6.24	*ACT*	7.00	*ACT*	7.00	*ACT*	7.00	*GADPH*	6.24

Table 2. Expression stability of candidate reference genes in small carpenter bee under different developmental stages and landscapes using reffinder analysis.

RefFinder analysis

RefFinder is an integrated analysis tool used for the validation of reference genes that incorporates several methods including GeNorm, BestKeeper, NormFinder, and ΔCt analysis. Based on the RefFinder, *RPL8* and *RPS18* were the top two most sable genes in the larval and adult stages. *RPS18* consistently ranked among the most stable candidate reference genes across all collection sites, paired with *EF-1α* in the organic site, *RPS5* in the roadside site and *RPL8* in the conventional site (Table 2).

Discussion

C. calcarata is considered an indicator species of healthy ecosystems and an important pollinator for natural and agricultural ecosystems[45,46]. This species is commonly used for studying pollinator ecology, behavior, evolution and genomics[17,47]. In this study, we collected larval and adult stages of *C. calcarata* from different habitats, namely organic, roadside and conventional landscapes and evaluated the expression stability of seven candidate reference genes as *ACT*, *EF-1α*, *GADPH*, *RPL8*, *RPL32*, *RPS5* and *RPS18* using widely adopted analytical tools. Integrated analysis using RefFinder revealed that *RPS18* consistently ranked among the top two most sable genes across both developmental stages and all habitat types. Similarly, *RPS5* was identified as one of the two most stable genes in larvae, adults and roadside habitat. However, *EF-1α* and *RPL8* were among the other two most stable genes in the organic and conventional site, respectively. In a study of a solitary bee, *Megachile rotundata*, also reported *RPS18*, and *RPL8* as stable reference genes across all life stages and under a variety of environmental conditions[48]. Similarly, a transcriptional study of *C. calcarata* reported significant variation in gene expression associated with overwintering[27]. These findings suggest that *RPS18* and *RPL8* exhibit overall high expression stability and are suitable reference genes for gene expression studies in *C. calcarata* across different developmental stages and habitat conditions. To our knowledge, no study has investigated the gene expression stability of *C. calcarata*. Therefore, the present findings will be an important basis for future studies in *C. calcarata* with broader implications for native wild bees.

The expression stability of candidate reference genes varied across developmental stages and habitat types, suggesting that both intrinsic and environmental factors influence gene expression. In other bee species, such as *Euglossa viridissima* also exhibited age-related gene expression patterns[49]. Differences between larvae and adults may reflect distinct physiological processes, such as growth and differentiation in larvae and processes like reproduction, foraging or immune function in adults[50]. Additionally, environmental stressors such as pesticide exposure and resource availability may affect gene expression in bees[51]. These findings highlight the

need to carefully validate reference genes across both developmental stages and ecological contexts for accurate normalization in q-RT-PCR studies.

In conclusion, the present study evaluated the expression stability of seven candidate reference genes in *C. calcarata* across different developmental stages and habitat types. The results demonstrate that gene stability varies with both developmental stages and environmental conditions, underscoring the importance of selecting appropriate reference genes for accurate normalization in q-RT-PCR. Our findings provide a valuable resource for future gene expression studies in wild bees and highlight the necessity of validating reference genes under specific conditions.

Data availability

All raw data and related metadata of this report was deposited in Zenodo, [https://doi.org/10.5281/zenodo.16414347](https:/doi.org/10.5281/zenodo.16414347).

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Declarations

Competing interests

The authors declare no competing interests.

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